

Original article

Liver X receptor agonist prevents the evolution of collagen-induced arthritis in mice

Min-Chan Park¹, Yong-Jin Kwon¹, Soo-Jin Chung¹, Yong-Beom Park¹ and Soo-Kon Lee¹

Abstract

Objective. Liver X receptors (LXRs) have been characterized as regulators of macrophage inflammatory pathways. Synthetic LXR agonists inhibit the macrophage response to bacterial pathogens and antagonize the induction of a number of pro-inflammatory genes. The aim of this study was to investigate the preventive effects of synthetic LXR agonist, GW3965, treatment on the evolution of arthritis and inflammatory response in a murine CIA model.

Methods. Intradermal injection of bovine type II CIA in DBA/1 mice. Along with the induction of CIA, mice were treated with oral GW3965 (0.1, 0.3 or 1.0 mg/kg/day) or vehicle from Day 1 to Day 40. Clinical assessment for arthritis scores and histopathological assessment of joint sections were performed. The expression of inflammatory mediators was evaluated by immunohistochemical staining. Serum pro-inflammatory cytokine levels were determined using ELISA.

Results. The CIA incidence was 100% on Day 27 and the severity progressed until Day 35 with histological features of cartilage erosion in vehicle-treated mice. GW3965 treatment significantly reduced the arthritis incidence and attenuated the clinical and histological severity, compared with vehicle-treated mice. GW3965 treatment also significantly reduced inflammatory mediator production in joint sections and serum pro-inflammatory cytokine levels in a dose-dependent manner.

Conclusions. These results indicate that activation of LXRs suppresses the onset of CIA and reduces inflammation and joint destruction in CIA mice. The data could suggest that LXR treatment is an effective prophylactic approach to suppress the evolution of synovitis and resultant joint destruction observed in RA.

Key words: Rheumatoid arthritis, Liver X receptors, GW3965, Collagen-induced arthritis.

Introduction

RA is an autoimmune disease characterized by chronic inflammation and cartilage and bone destruction in the joints. The inflammation of RA results from the production of prostaglandins, pro-inflammatory cytokines and nitric oxide. In particular, in the pathogenesis of rheumatoid synovitis and synovial tissue, macrophages produce a number of inflammatory mediators and interact with

other cells or extracellular matrix macromolecules. In addition, macrophages are involved in the oxidative and nitrosative damage of the joints and their products mediate synovial angiogenesis. Macrophage-derived cytokines, such as TNF- α and IL-1 β , which are abundant in synovial tissues and fluid from RA patients, are mitogens for synovial fibroblasts and osteoclasts. These cytokines, acting in both autocrine and paracrine fashions within the joint space, mediate long-term cartilage degradation and bone erosion [1–6].

Macrophages play essential roles in immunity and lipid homeostasis. They function to scavenge pathogens and apoptotic cells, and it is generally considered that sustained activation of these responses may precipitate pathological states. Macrophages use the nuclear-receptor superfamily, such as peroxisome proliferator-activated

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receptors and liver X receptors (LXRs). The nuclear receptor superfamily has diverse and important roles in regulating reproductive, homeostatic, inflammatory, immune and metabolic processes [7–10]. Previous studies have outlined the importance of LXRs (LXR- α , encoded by the gene *Nr1h3*, and LXR- β , encoded by the gene *Nr1h2*) in the macrophage cholesterol efflux pathway. LXRs are 'cholesterol sensors' that regulate the expression of genes involved in lipid metabolism in response to specific oxysterol ligands [11]. In macrophages, oxysterols may be derived from internalized oxidatively modified low density lipoproteins (oxLDL) or generated intracellularly from modified cholesterol [12, 13]. LXR activation in macrophages induces expression of several genes involved in lipid metabolism and reverse cholesterol transport, exerting an atheroprotective effect. In addition, LXRs have more recently been characterized as macrophage inflammatory pathway regulators. Mice lacking LXRs show an exaggerated response to lipopolysaccharide. Synthetic LXR agonists inhibit the macrophage-response to bacterial pathogens and down-regulate a number of pro-inflammatory genes. These include IL-1 β , IL-6, MMP-9, inducible nitric oxide synthase (iNOS), cyclo-oxygenase 2 (COX2), adhesion molecules and chemokines [14–16]. Some of these genes are preferentially sensitive to LXRs, indicating LXR-specific effects on inflammatory responses. LXR ligands repressed inflammatory gene expression in macrophages derived from wild-type, *Lxra*^{-/-} and *Lxrb*^{-/-} mice, but could not do so in cells from *Lxrab*^{-/-} mice, indicating that both LXR isoforms possess anti-inflammatory properties [17, 18]. The precise molecular mechanisms responsible for inhibiting inflammatory response genes by LXRs remain to be established, but are at least in part linked to nuclear factor- κ B (NF- κ B) inhibition [16, 17, 19].

Inflammatory rheumatic diseases, including RA, are significant risk factors in atherosclerosis and coronary heart disease [20, 21]. Furthermore, patients with active RA show altered lipid metabolism, which can be reversed by intensive anti-rheumatic treatment [22], and some of the shared inflammatory mechanisms responsible for rheumatoid synovitis are thought to be directly involved in producing atherosclerotic lesions [23, 24]. These observations suggest a strong integration between metabolism and inflammation. Because LXRs act as a cross-talk between lipid metabolism and inflammation, they may be beneficial in treating arthritis. The purpose of the present study is to investigate the prophylactic and disease-modifying effect of LXRs in a murine CIA, a model that mimics human RA clinically and histologically. We investigated whether a synthetic LXR agonist, GW3965, could prevent the evolution of murine CIA and suppress bone and cartilage destruction in CIA mice.

Materials and methods

CIA induction and treatment

All animals were housed according to the Animal Care Committee of Yonsei University College of Medicine

guidelines. All experimental protocols were reviewed and approved by the Animal Care Committee. Male DBA/1J mice at 8–10 weeks of age (SLC, Shizoka, Japan) were used for CIA induction. Bovine type II collagen (CII; Sigma Chemical, St Louis, MO, USA) was dissolved in 0.01 M acetic acid at 2 mg/ml by stirring overnight at 4°C. Freund's complete adjuvant was prepared by adding *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI, USA) at 5 mg/ml. Mice were injected intradermally at the base of the tail with 100 μ l of emulsion (containing 100 g of CII) on Days 1 and 21 as described previously [25]. All mice were fed with GW3965, a synthetic LXR agonist, or vehicle, beginning from Day 1 to Day 40. The synthetic LXR agonist, GW3965, was provided by Jon L. Collins (GlaxoSmithKline R&D, USA) and GW3965 treatment was performed in a manner similar to that in the previous studies [26, 27]. Briefly, mice were fed a western diet (Research diets, Central Lab. Animal Inc., Seoul, Korea; 21% fat, 1.5% cholesterol) supplemented with GW3965 at a dose of 0.1, 0.3 and 1.0 mg/kg/day or vehicle control (0.5% methylcellulose, 2% Tween-80) from Day 1 to Day 40.

Sham-treated mice were treated on Days 1 and 21 with 100 μ l of 0.01 M acetic acid. Sham-treated mice were also fed with GW3965 or vehicle (10% DMSO) from Day 1 to Day 40. Ten mice were assigned to each group.

Clinical and histological assessment of CIA

An investigator blinded to the treatment regimen performed all clinical examinations as previously described [28], using the following system: Grade 0, no swelling; Grade 1, slight swelling and erythema; Grade 2, moderate swelling and oedema; Grade 3, extreme swelling and pronounced oedema; and Grade 4, joint rigidity. Mice were evaluated for arthritis on a daily basis. Each limb was graded and limb scores added, giving a maximum possible score of 16 per animal. For histological analysis, mice were anaesthetized and sacrificed 40 days after primary immunization. Paws were randomly collected and fixed in 4% buffered formaldehyde, decalcified, paraffin-embedded, sectioned and stained with haematoxylin and eosin (H&E). Histopathological changes were scored in a blinded manner based on cell infiltration, cartilage destruction and bone erosion parameters, as previously described [29]. Minor differences between observers were resolved by mutual agreement.

Immunohistochemical localization of inflammatory mediators

Mice were anaesthetized and sacrificed on Day 40, and paws and knee joints were removed for histopathological examination, which was performed after fixation, decalcification and paraffin embedding of tissue sections. Tissue sections were prepared and stained with H&E.

Sections were sequentially incubated with specific antibodies directed against murine TNF- α , IL-1 β , iNOS or COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by the appropriate secondary antibodies (ISU Abxis, Seoul, Korea). All tissue samples were counterstained with haematoxylin. After immunohistochemical

staining, expression of different markers in the joint synovial tissue was scored semi-quantitatively on a four-point scale, independently and blindly by two investigators, and the averages were calculated. A score of 0 represented minimal expression, 1 represented mild expression, 2 represented moderated expression, whereas 3 represented abundant marker expression [30].

Measurement of pro-inflammatory cytokine levels

The TNF- α , IL-1 β and IL-6 levels in serum from CIA mice were determined by specific ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Both intra- and interassay coefficients of variation were <8% for all assays.

Assessments for GW3965 toxicity

We used 15 CIA mice for investigation of the toxicity of GW3965: controls and CIA mice treated with 0.3 and 1.0 mg/kg of GW3965. We obtained blood from the controls and the mice treated with GW3965 (0.3 and 1.0 mg/kg) after sacrifice, and examined white blood cell count, haemoglobin, platelet count and levels of aspartate

aminotransferase, alanine aminotransferase, blood urea nitrogen and creatinine for liver and kidney toxicity.

Statistics

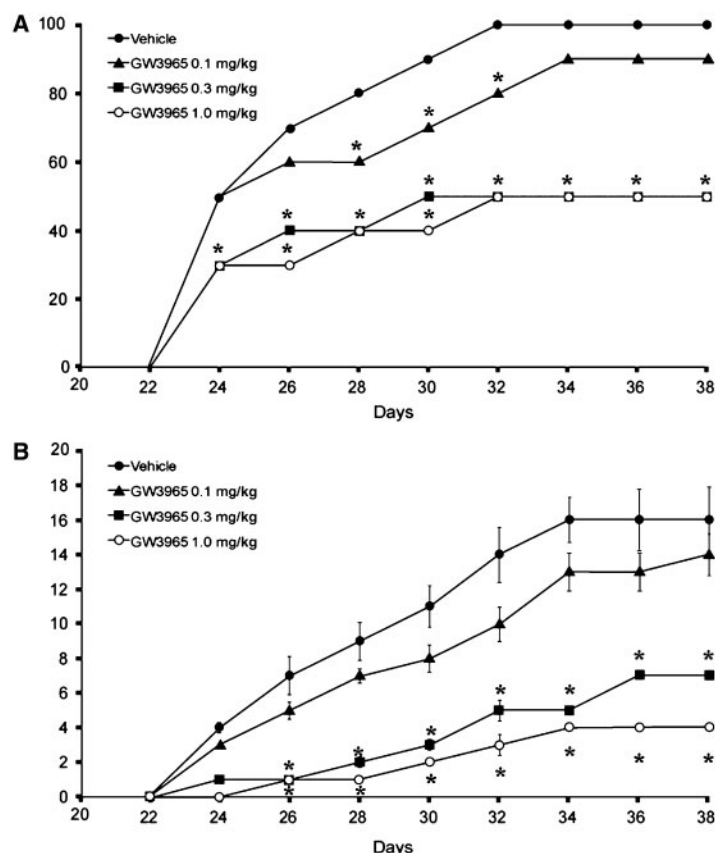
The results are shown as the mean (s.e.m.). In experiments involving histology or immunohistochemistry, the results are representative of at least three experiments performed on different days. Data were examined by one-way analysis of variance followed by a Bonferroni *post hoc* test for multiple comparisons. A Mann-Whitney U-test was used to compare the arthritis index medians. $P < 0.05$ was considered significant.

Results

Effect of GW3965 treatment on CIA evolution

CIA developed rapidly in mice immunized with CII. Clinical signs of the disease, including periarticular erythema and oedema, first appeared in the hind paws between 23 and 24 days after CII immunization, with a 100% CIA incidence by Day 32 (Fig. 1A). Erythema and swelling in the hind paws increased in frequency and severity in a

Fig. 1 Effects of GW3965 on arthritis onset and severity in CIA mice.



(A) The percentage of arthritic mice (arthritis scores >1) is significantly different between vehicle and GW3965 treatment. Treatment with 0.1 mg/kg/day GW3965 slightly suppressed arthritis onset, whereas 0.3 and 1.0 mg/kg/day GW3965 significantly reduced arthritis incidence. (B) Median arthritis scores also indicate a significant difference between vehicle and GW3965 treatment. Doses of 0.3 and 1.0 mg/kg/day of GW3965 significantly reduced arthritis incidence * $P < 0.05$.

time-dependent manner, reaching maximum arthritis indices between 32 and 34 days after CII immunization in the vehicle-treated mice (Fig. 1B). GW3965 treatment suppressed the evolution of CIA in a dose-dependent manner, demonstrated by lower incidence and reduced arthritis severity as shown in Fig. 1. A 0.1 mg/kg/day dose of GW3965 also decreased arthritis severity significantly, but did not remarkably suppress arthritis onset, compared with 0.3 and 1.0 mg/kg/day doses. There was no macroscopic evidence of either hind paw erythema or oedema in the sham-treated mice (data not shown).

On Day 40, histological evaluation of paws from CII-immunized mice revealed signs of severe arthritis and bone erosion. A comparison of the histological damage scores is presented in Fig. 2. GW3965 treatment reduced inflammatory cell infiltration and bone destruction in CIA mice (Fig. 2A). A 0.3 and 1.0 mg/kg/day GW3965 treatment significantly reduced the histological damage scores in the joint sections, compared with vehicle-treated mice. However, no significant reduction in arthritis was observed in the mice treated with 0.1 mg/kg of GW3965. There was no evidence of disease in the sham-treated mice from either treatment group (Fig. 2B).

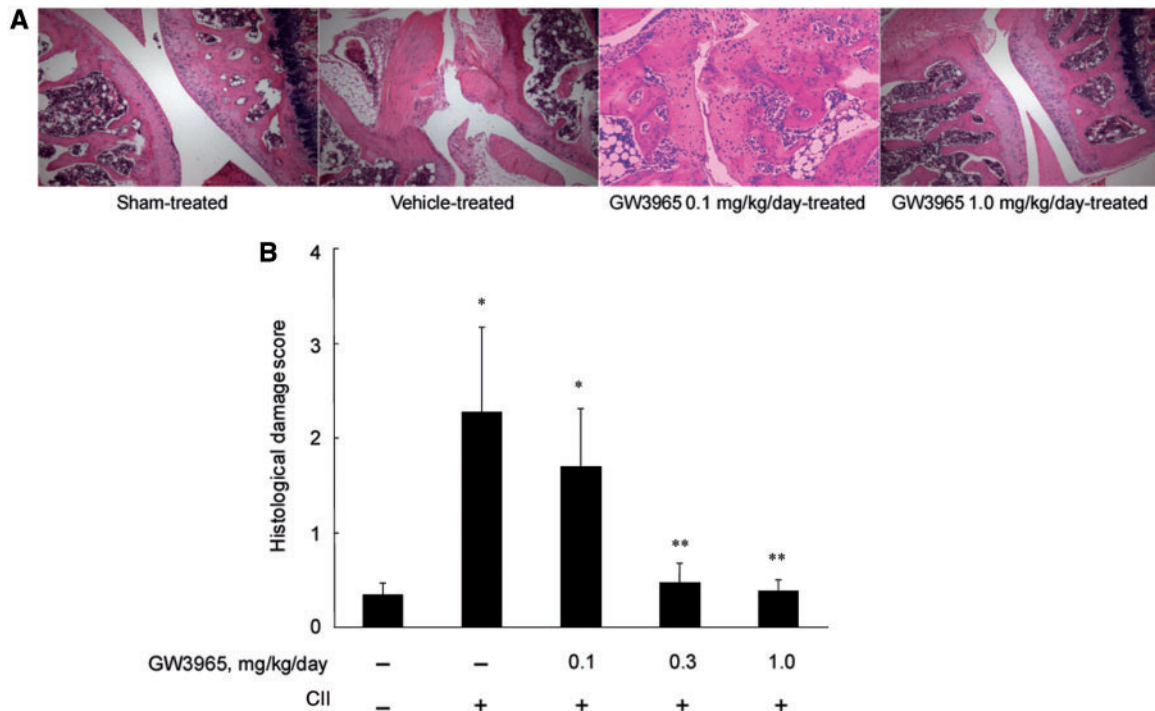
Effect of GW3965 treatment on TNF- α , IL-1 β , iNOS and COX-2 production

Figure 3 shows results from immunohistochemical analysis of joint sections representative of at least two experiments performed on different days. Joints obtained from vehicle-treated mice showed positive staining for TNF- α , IL-1 β , iNOS and COX-2, localized primarily to inflamed cells around the joints. GW3965-treated mice with CIA showed significantly reduced staining (Fig. 3A). Semi-quantitative analysis demonstrated a significant dose-dependent decrease in TNF- α , IL-1 β , iNOS and COX-2 stain positivity in GW3965-treated mice, compared with vehicle-treated mice. There was no TNF- α , IL-1 β , iNOS or COX-2 staining around joints obtained from sham-treated mice (Fig. 3B).

Effect of GW3965 treatment on pro-inflammatory cytokine levels

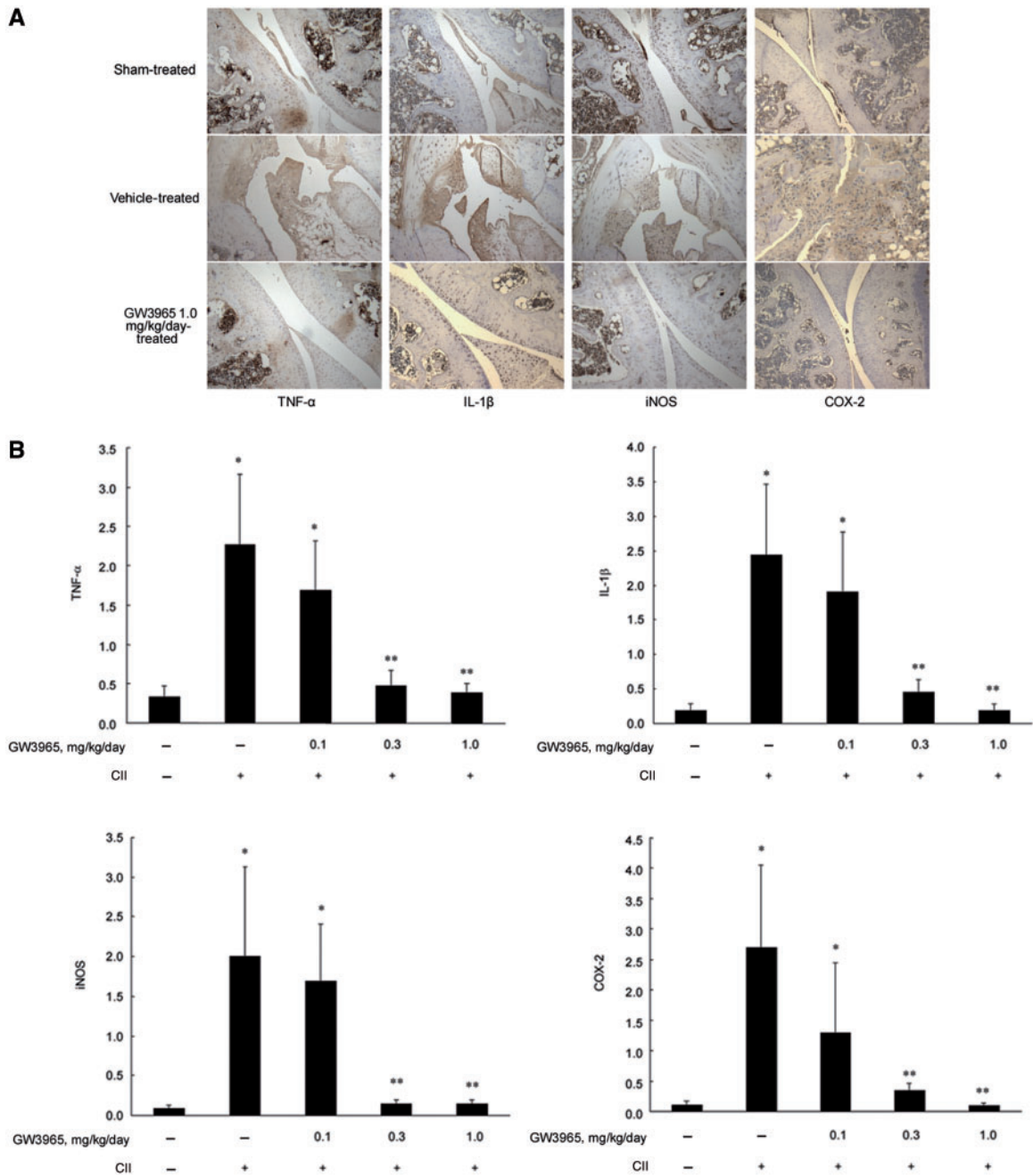
The pro-inflammatory cytokine expression pattern, as assessed by ELISA, is shown in Fig. 4. Compared with vehicle-treated mice, GW3965-treated mice had lower serum TNF- α , IL-1 β and IL-6 levels. Serum TNF- α levels in vehicle-treated and 0.1 mg/kg/day GW3964-treated mice were not significantly different,

Fig. 2 Effects of GW3965 on histological changes.



(A) Joint sections from paws of vehicle-treated mice show inflammatory cell infiltration and bone erosion, but remarkably reduced inflammatory cell infiltration in GW3965-treated mice (original magnification $\times 100$). (B) Treatment with 0.3 and 1.0 mg/kg/day of GW3965 significantly reduced the histological damage scores in CIA mice. Values are mean (S.E.M.) of 10 mice per group. * $P < 0.01$ vs sham-treated control; ** $P < 0.01$ vs vehicle-treated CIA mice.

Fig. 3 Effects of GW3965 treatment on immunohistochemical staining for TNF- α , IL-1 β , iNOS and COX-2.

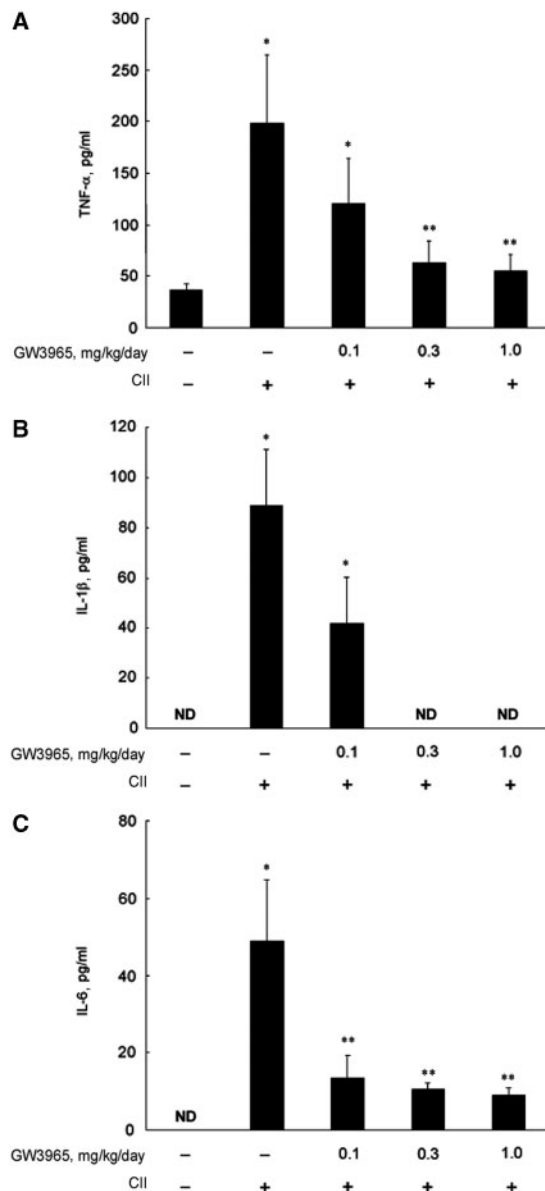


(A) Vehicle-treated mice showed positive staining for TNF- α , IL-1 β , iNOS and COX-2, which was localized primarily to inflamed cells around the joints. Staining was significantly reduced in GW3965-treated CIA mice. There was no specific staining in joints from sham-treated mice (original magnification $\times 100$). **(B)** Treatment with 0.3 and 1.0 mg/kg/day of GW3965 significantly reduced TNF- α , IL-1 β , iNOS and COX-2 expression. * $P < 0.01$ vs sham-treated control; ** $P < 0.01$ vs vehicle-treated mice with CIA.

but they were significantly greater than in 0.3 and 1.0 mg/kg/day GW3965-treated mice. IL-1 β and IL-6 serum levels in vehicle-treated mice were significantly higher than those in all GW3965-treated mice, but the

differences between GW3965 doses were not statistically significant. There were no significant increases in serum levels of any of the three cytokines in sham-treated mice.

Fig. 4 Effects of GW3965 treatment on pro-inflammatory cytokine production.



GW3965 treatment significantly reduced TNF- α (A), IL-1 β (B) and IL-6 (C) serum levels in CIA mice compared with vehicle-treated mice. * $P < 0.01$ vs sham-treated control; ** $P < 0.01$ vs vehicle-treated mice with CIA. ND: not detected.

Toxicity of GW3965 in CIA mice

Table 1 shows the results of laboratory tests performed to investigate the toxicity of GW3965. There were no differences in white blood cell counts, haemoglobin levels and platelet counts between the controls and the mice treated with GW3965 (0.3 and 1.0 mg/kg). Liver and kidney toxicities were not observed in the mice treated with 0.3 and 1.0 mg/kg of GW3965.

Discussion

The present study investigated the potential usefulness of a synthetic LXR agonist, GW3965, as an anti-inflammatory agent to suppress arthritis in CIA mice, and found that GW3965 suppresses CIA incidence and severity. Higher GW3965 doses (0.3–1.0 mg/kg/day) reduced CIA incidence up to 50% compared with vehicle-treated mice. Although a 0.1 mg/kg/day GW3965 treatment also significantly decreased CIA incidence, the effect was observed at a relatively late time point, and disappeared with time in comparison with higher GW3965 doses. In addition to suppressing CIA onset, GW3965 treatment significantly reduced CIA severity. GW3965 treatment significantly reduced the median arthritis indices and histological joint damage, compared with vehicle-treated mice. Both effects were more prominent at higher GW3965 doses.

The regulatory action of LXRs in inflammatory responses is well understood. In addition to the originally identified atheroprotective effects [31, 32], LXRs have recently been identified as macrophage inflammatory pathway regulators. Synthetic LXR agonists, such as GW3965 and T0901317, antagonise the expression of pro-inflammatory genes, such as iNOS, COX2, IL-6, IL-1 β , monocyte chemoattractant protein-1 (MCP-1), MCP-3 and MMP9 [17–19]. The precise mechanisms by which LXRs exert these anti-inflammatory effects remain to be established, but a body of evidence implicates inhibition of the NF- κ B pathway. Cytokine or bacterial component recognition by the corresponding receptor initiates pro-inflammatory gene expression, and toll-like receptor 3/4 activation by these signals blocks LXR-dependent gene transcription. Moreover, ligand activation of LXR inhibits inflammatory responses to cytokines via a blockade of NF- κ B signalling [15, 17, 19, 33]. In the rheumatoid synovium, activation of the NF- κ B pathway induces various genes that contribute to the inflammatory response, such as TNF- α , IL-1 β , COX-2, MMPs and chemokines that recruit immune cells to the inflamed pannus [34–36]. The present study demonstrated that GW3965 treatment significantly reduced TNF- α , IL-1 β , iNOS and COX-2 expression in the joint tissue and serum pro-inflammatory cytokine levels in CIA mice. Thus, the suppressed CIA incidence and severity by GW3965 treatment might result, at least in part, from NF- κ B pathway inhibition. Furthermore, GW3965 may serve as a negative regulator of articular inflammation in CIA mice.

LXR- α is highly expressed in liver, adipose tissue and macrophages, whereas LXR- β is expressed ubiquitously. Recently, Chintalacharuvu *et al.* [37] showed both LXR- α and LXR- β overexpression in synovium from RA patients, but not in normal synovium. Although the therapeutic effect of LXR agonists in established arthritis has not been reported in the literature, the recent work by Chintalacharuvu *et al.* [37] documented the regulatory effect of a synthetic LXR agonist, T0901317, on pro-inflammatory cytokine production and resultant joint damage in lipopolysaccharide-treated mice [37]. These results support the proposal that LXR agonists might

TABLE 1 Laboratory findings in control and GW3965-treated mice

Parameters	Control	GW3965 0.3 mg/kg	GW3965 1.0 mg/kg
White blood cell, per μ l	8905.5 (1003.2)	8555.3 (975.1)	9040.0 (716.5)
Haemoglobin, g/dl	13.8 (0.5)	13.6 (0.5)	13.3 (0.5)
Platelet, $\times 1000/\mu$ l	605.2 (51.0)	555.1 (77.5)	531.2 (80.2)
Blood urea nitrogen, mg/dl	24.5 (3.2)	27.1 (3.0)	26.6 (2.5)
Creatinine, mg/dl	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)
Aspartate transaminase, IU/l	141.1 (34.0)	128.9 (31.0)	145.5 (22.3)
Alanine transaminase, IU/l	68.9 (17.0)	78.8 (14.4)	79.1 (11.6)

All results are expressed as mean (s.d.).

prove clinically useful in suppressing autoimmune diseases, in particular RA. The present study used CIA mice, which has proved to be a useful RA model, since it possesses many cellular and humoral immunity characteristics found in human RA [38, 39]. Similar to previous observations, GW3865 treatment, in the present study, significantly reduced the degree of joint damage in CIA mice by decreasing the expression of inflammatory mediators in a dose-dependent manner. The most interesting finding was that higher LXR agonist doses significantly suppress the evolution of arthritis in CIA mice, which indicates a prophylactic effect of LXR agonist against arthritis onset.

Although the function of LXRs to induce macrophage cholesterol efflux and inhibit inflammatory gene expression could generate widespread interest in these proteins as potential targets for atherosclerosis and autoimmune disease treatment, synthetic LXR agonists can increase triglyceride levels. LXR- α is the predominant LXR expressed in liver, and the ability of LXR agonists to stimulate hepatic lipogenesis likely results primarily from LXR- α activation [40–42]. In this regard, the present study has some potential limitations: (i) we evaluated the prophylactic effect of a non-selective, not isotype-selective, synthetic LXR agonist on the evolution of CIA in mice and (ii) we demonstrated only the prophylactic action of GW3965 on the development of CIA and its therapeutic effects on established disease was not evaluated. The demonstration of prophylactic effect alone could not be sufficient for the clinical significance of LXR agonist for the treatment of RA. Thus, the present study mandates future investigations to define the roles of tissue- or isotype-selective LXR agonists in the RA inflammatory response and to investigate the therapeutic effect of synthetic LXR agonists on established disease.

In conclusion, treatment with a synthetic LXR agonist significantly suppressed arthritis incidence and attenuated its severity in CIA mice. This LXR agonist reduced the degree of joint damage by inhibiting the expression of pro-inflammatory mediators. The data obtained in the present study support the conclusion that LXR activation

exerts a beneficial effect in RA, given its anti-inflammatory properties, and could serve as a novel prophylactic modality for RA.

Rheumatology key messages

- GW3965 prophylaxis significantly reduced arthritis incidence and attenuated the clinical and histological severity in CIA mice.
- GW3965 prophylaxis significantly reduced inflammatory mediator production in joint sections and serum pro-inflammatory cytokine levels in a dose-dependent manner.

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